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Recognition of the Peripheral Self by Naturally Arising CD25$^+$ CD4$^+$ T Cell Receptors

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Summary

Naturally arising CD25$^+$ CD4$^+$ regulatory T cells (T_R) play an important role in the prevention of autoimmunity. TCR specificity is thought to play a critical role in T_R development and function, but the repertoire and specificity of T_R TCRs remain largely unknown. We find by sequencing of TRAV14 (Vα2) TCRα chains associated with a transgenic TCRβ chain that the T_R and CD25$^-$ CD4$^+$ TCR repertoires are similarly diverse, yet only partially overlapping. Retroviral expression of TCRα genes in TCR transgenic RAG-deficient T cells revealed that a high frequency of TCRs derived from CD25$^+$ but not CD25$^-$ CD4$^+$ T cells confers the ability to rapidly expand upon transfer into a lymphopenic host. Thus, these data show that a large proportion of naturally arising T_R have substantially more efficient interactions with MHC class II bound peptides from the peripheral self than CD25$^-$ T cells.

Introduction

Naturally arising CD25$^+$ CD4$^+$ regulatory T cells (T_R) play an important role in the preservation of self-tolerance by suppressing autoreactive T cells in the periphery (reviewed in Maloy and Powrie, 2001; Sakaguchi, 2000; Shevach, 2002). Several lines of evidence suggest that T_R development and function hinges upon a particular TCR specificity. First, T_R do not develop in TCR transgenic mice lacking RAG genes (Itoh et al., 1999), suggesting that only cells with certain TCR specificities can develop into T_R. Second, TCR engagement is required for T_R suppression in both in vitro (Takahashi et al., 1998; Thornton and Shevach, 1998) and in vivo (Apostolou et al., 2002; Klein et al., 2003) models. Thus, there are abundant data demonstrating that TCR recognition of specific ligands is critical for naturally arising T_R development and function.

In contrast, there are only sparse data characterizing the TCR repertoire of regulatory T cells. It appears that T_R utilize a polyclonal TCR repertoire that is not severely restricted, as Vα and Vβ usage is similar between CD25$^+$ and CD25$^-$ CD4$^+$ T cells (Takahashi et al., 1998). However, the actual diversity of the T_R TCR repertoire is unknown. Furthermore, the relationship between the T_R and CD25$^-$ TCR repertoires is unclear (Shevach, 2002).

Information on the antigen specificity of T_R TCRs is also lacking. The current paradigm is that T_R develop due to TCR interactions with cognate self-peptide:MHC class II complexes in an avidity range between positive and negative selection (reviewed in Maloy and Powrie, 2001). This view is based primarily on a single TCR transgenic model of T_R development where the cognate peptide was also expressed as a transgene (Jordan et al., 2001). In this experimental system, a high level of peptide expression resulted in the deletion of TCR transgenic T cells, whereas a moderate level resulted in partial deletion, with up to 50% of the remaining peripheral T cells expressing CD25 and exhibiting functional properties resembling naturally arising T_R. This model would therefore predict that the CD25$^+$ and CD25$^-$ TCR repertoires are distinct and that the former is comprised of self-reactive TCRs.

However, one potentially serious caveat of the above experiments is that the transgene-enforced high-affinity TCR recognition of cognate peptide may not represent the mechanism by which naturally arising T_R develop. For example, this high-affinity TCR signal may mimic unknown "non-TCR"-mediated signals, such as cytokines, required for naturally arising polyclonal T_R development. Recent papers have further questioned the applicability of this model to naturally arising T_R. First, it has been demonstrated that naturally arising CD25$^+$ T_R can recognize foreign antigens such as 2,4-dinitrofluorobenzene (Dubois et al., 2003), *Candida albicans* (Montagnoli et al., 2002), and *Leishmania major* (Belkaid et al., 2002). Second, at least some peripheral CD25$^-$ T cells can be converted into CD25$^+$ T cells with regulatory properties in both human (Walker et al., 2003b) and murine (Chen et al., 2003; Thorstenson and Khoruts, 2001) models. Last, recent attempts to evaluate T_R self-reactivity utilizing in vitro limiting dilution or in vivo CFSE dilution assays were confounded by the sensitivity of the assays and by the heterogeneity of T cells in regards to expression of adhesion molecules, cytokine receptors, etc. (Fisson et al., 2003; Romagnoli et al., 2002). Thus, the question of self-reactivity within the naturally arising CD25$^+$ T_R population remains unresolved.

In order to directly address this issue, we analyzed the TCR repertoires of naturally arising CD25$^+$ and CD25$^-$ T cell subsets and tested their ability to interact with self-peptide:MHC class II complexes. For our studies, we utilized the TCR repertoire represented by variable TRAV14 (Vα2) TCRα chains paired with a fixed TCRβ chain. We find by sequencing that the T_R and CD25$^-$ TCR repertoires are diverse and only partially overlapping. The specificity of the cloned T_R TCRs was then analyzed both in vitro and in vivo by retrovirus-mediated expression in mono-specific TCR$\alpha\beta$ transgenic RAG-deficient T cells. Using this approach, we demonstrate that a large proportion of naturally arising T_R TCRs recognize constitutively presented peripheral self-antigens with higher efficiency than CD25$^-$ TCRs.

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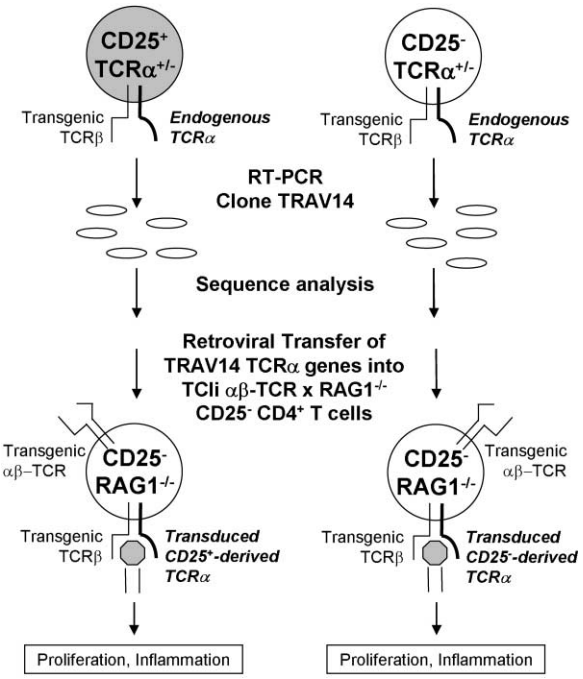

Figure 1. Experimental Strategy

TCRβ transgenic mice develop naturally arising $CD25^+$ $CD4^+$ T cells that are anergic/hypoproliferative and suppressive upon TCR ligation. Because the TCRβ chain is fixed, the TCR repertoire can be analyzed by cloning and sequencing only the endogenously rearranged TCRα genes from $CD25^+$ or $CD25^-$ $CD4^+$ TCli TCRβ transgenic T cells. As the mice are on a $TCR\alpha^{+/-}$ background, only one TCR is expressed per T cell. TCRα genes can then be retrovirally expressed in $CD25^-$ $CD4^+$ αβ-TCR transgenic × $RAG1^{-/-}$ T cells, recreating the original TCR. TCR signaling in the recipient $CD25^-$ T cell should result in conventional T cells responses such as proliferation, irrespective of the origin of the transferred TCR.

Results

The T_R and $CD25^-$ $CD4^+$ TCR Repertoires Are Diverse and Show Only Limited Overlap

Previous studies have not found any differences in Vα or Vβ gene usage between the $CD25^+$ and $CD25^-$ subsets (Takahashi et al., 1998). However, it is unknown whether T_R have a limited or unrestricted TCR repertoire and whether this repertoire is distinct from, or largely overlapping with, the $CD25^-$ $CD4^+$ TCR repertoire. To address these questions, we investigated the TCR repertoire displayed by $TRAV14^+$ ($V\alpha2$) $CD4^+$ T cells in TCli TCRβ transgenic mice (Figure 1). These T cells represent ~10% of both the peripheral $CD25^+$ and $CD25^-$ subsets. In previous work, we found that significant numbers of T_R develop in these transgenic mice only in the presence of endogenous TCRα rearrangement (Gavin et al., 2001). To exclude from our analysis two productively rearranged TCR genes expressed in a single cell, we used TCli TCRβ transgenic mice heterozygous for the Cα null mutation (Mombaerts et al., 1992). We believe that fixing the TCRβ chain to study the T_R TCR repertoire is a valid approach for the following two reasons. First, limiting the TCR repertoire in this manner has been invaluable for direct TCR repertoire analysis by sequencing (Correia-Neves et al., 2001). Second, the use of a single TCRβ chain should not adversely affect $CD25^+$ T cell development. This notion is supported by numerous observations in TCR transgenic mice that functional T_R develop only with the expression of endogenous TCR chains (Apostolou et al., 2002; Itoh et al., 1999; Thornton and Shevach, 2000). As TCRβ allelic exclusion is very efficient, these data strongly suggest that the endogenously rearranged TCRα chains are largely responsible for the antigen specificity and development of T_R when paired with the transgenic TCRβ chain.

We sequenced over 600 TRAV14 TCRα clones isolated from the $CD25^+$ and $CD25^-$ subsets in two independent experiments (Table 1). Pooled T cells from two mice were used for each experiment. TCR sequences were analyzed by IMGT-V-QUEST (IMGT, the international ImMunoGeneTics information system, at http://imgt.cines.fr; initiator and coordinator, Marie-Paule Lefranc, Montpellier, France). Twelve different subregions of TRAV14 and 41 distinct J regions were found in these TCR sequences. Interestingly, there was some consistent skewing in both TRAV14 subtype and J region usage between the T_R and $CD25^-$ subsets (Figure 2A). For example, TRAJ7 was 6-fold more common in $CD25^+$ T cells, whereas TRAV14-2*02 was 2-fold more common in $CD25^-$ T cells. Although there were a number of similarities between the T_R and $CD25^-$ subsets, these results hinted at substantial differences in the TCR repertoires of T_R and $CD25^-$ $CD4^+$ T cells.

To further explore this possibility, we analyzed TCRα CDR3 amino acid sequences, as this region of the TCR makes the greatest contribution to antigen specificity. Initial examination of the CDR3 length revealed a Gaussian distribution, primarily in the range of 10 to 13 amino acids for both the T_R and $CD25^-$ subsets (Figure 2B). Furthermore, the distribution of the number of TCRs with a unique CDR3 sequence appeared to be statistically similar between the $CD25^+$ and $CD25^-$ subsets (Table 2). These data therefore suggest that both subsets have a diverse TCR repertoire displaying few predominant clones.

To estimate the total number of unique TCR sequences at the CDR3 level, we utilized previously described statistical approaches for evaluating the diversity of biological species (ACE) or TCRs (MLE) from a random sampling (Casrouge et al., 2000; Colwell, 1997). These statistical approaches also suggest that the T_R TCli-β TCR repertoire is as diverse as the $CD25^-$ repertoire, containing an estimated 125–300 unique TRAV14 CDR3s at the amino acid level (Table 1).

Any number derived from an approach based on random sampling may be an underestimate if the $CD25^+$ and/or $CD25^-$ T cell populations contain an infrequent yet very diverse subset of TCRs. Nevertheless, the equivalent diversity observed between the $CD25^-$ and $CD25^+$ TCRs is unlikely to result from an artifact of our cloning strategy for several reasons. First, the use of bulk PCR-based techniques for estimating TCR diversity has been previously validated (Casrouge et al., 2000). Second, we utilized over 4-fold more $CD25^-$ than $CD25^+$ T cells for mRNA isolation, indicating that the amount of starting material should not limit the observed diversity of the $CD25^-$ TCR library relative to the $CD25^+$ library. Third, the abundance coverage estimator (ACE) appeared to reach a plateau when plotted as a function

Table 1. Summary of TRAV14 TCRα Sequence Sets and Analysis of Diversity Using CDR3 a.a. Sequence to Identify Unique TCRs

Experiment	Source	Total No. of Sequences Sampled (n)	No. of Unique CDR3 a.a. Sequences (M)	Estimated No. of Unique CDR3s in Original Popl. (ACE ~N)	Estimated No. of Unique CDR3s in Original Popl. (MLE ~ N (95% CI))
1	CD25+	127	94	285	198 (158,281)
	CD25−	123	82	270	138 (116,186)
2	CD25+	149	108	346	217 (177,293)
	CD25−	148	87	297	125 (108,154)

TCRα chains were cloned and sequenced from purified CD25+ or CD25− CD4+ T cells as described in the text. The estimated number of total unique sequences (~N) is derived from an abundance coverage estimator (ACE, calculated by the EstimateS program [Colwell, 1997]), which is based on the probability of sampling a particular species multiple times and thus derived from Table 2, or the MLE, which is based only on the number of unique sequences in the sample set (M) shown in this table and has been previously used to estimate TCR diversity (Casrouge et al., 2000). Both the ACE and MLE are based on the probability that random sampling would generate the observed distribution of singletons, doubletons, and so forth, of distinct isolates. However, the ACE, unlike the MLE, does not assume that all individual species are equally distributed. We considered a particular CDR3 amino acid sequence a unique "species" for this purpose.

of the size of the TCR sample set (data not shown), suggesting that the number of TCRs sequenced is adequate to reach the semiquantitative conclusion that the TCR repertoire of CD25+ and CD25− CD4+ T cells are comparably diverse. Last, our estimates of TCR diversity are in the range of previous reports using limited TCR repertoire T cells (Correia-Neves et al., 2001). Taken together, the data on the distribution of TCRs (Table 2) and the direct estimation of the TCR population (Table 1) argue for a remarkable diversity within the CD25+

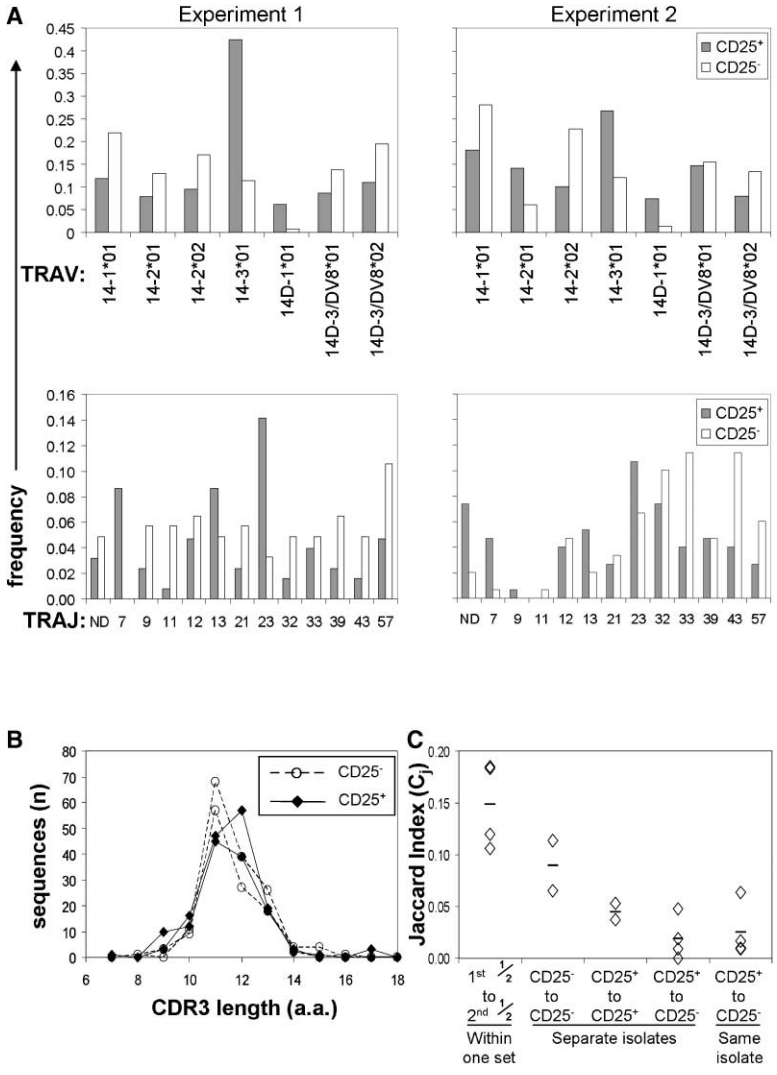

Figure 2. The T_R TRAV14:TCli-β TCR Repertoire Only Partially Overlaps with the CD25− CD4+ Repertoire

TRAV14 TCRα genes were isolated by RT-PCR from CD25+ and CD25− T cells purified from two TCli-TCRβ × TCRα+/− mice as described in the Experimental Procedures on two different occasions to generate two independently derived CD25+ and CD25− TCRα libraries. TCRα gene sequences were analyzed by IMGT-V-QUEST.

(A) TRAV14-subtype and J region usage by CD25+ and CD25− T cells. Data shown are for V subtypes and J regions with a greater than 5% and 2% frequency, respectively. Results of two independent experiments are shown. ND (not determined) refers to sequences for which IMGT did not identify a TRAJ region.

(B) CDR3 length distribution as determined by IMGT-V-QUEST.

(C) Similarity Index analysis suggests that the T_R and CD25− TCR repertoires overlap by less than ~25%. The Jaccard index is an estimate of the similarity between two sample sets (C_j = number of common species/number of total species observed). The number of occurrences of each sampled species is not considered. For this purpose, an individual TCR species is defined by its unique CDR3 amino acid sequence. By comparing the first half of each CDR3 sequence sample set with the second half, we obtained an empiric index value for sample sets that should be identical (~0.15). We then compared the first half of each set with the first half of other sets, as well as the second half with the second half. The average Jaccard index of ~0.025 between CD25+ and CD25− sets isolated at the same time, in combination with data from the Morisita-Horn index calculations (Supplemental Figure S1), suggests that the T_R and CD25− TCR repertoires overlap semiquantitatively by <25%. Jaccard and Morisita-Horn indexes were calculated using the statistical package EstimateS (Colwell, 1997).

Table 2. Frequency of Individual TCRs with Unique CDR3 Amino Acid Sequences

No. of Times an Individual CDR3 a.a. Sequence Is Found within a Sequenced Set	No. of Unique CDR3 Sequences at Given Frequency			
	Experiment 1		Experiment 2	
	$CD25^+$	$CD25^-$	$CD25^+$	$CD25^-$
1	72	63	83	65
2	17	8	15	9
3	1	5	8	6
4	2	2		2
5	2	3	1	2
6		1		
7			1	1
8				1
14				1

The distribution of TCRs between the $CD25^+$ and $CD25^-$ subsets within an experiment are statistically comparable, suggesting similar diversity, as assessed by Fisher's Exact Test. This approach allows for an evaluation of relative diversity without attempting to directly assess the number of species within the given population and provides exact p values in finite samples. The p values of 0.20 and 0.58 obtained for Experiments 1 and 2, respectively, are consistent with the null hypothesis that the two distributions are the same, suggesting an equivalent or comparable diversity between the $CD25^+$ and $CD25^-$ subsets.

TCR repertoire, which appears to be comparable to the $CD25^-$ TCR repertoire.

Next, we tested the degree of overlap between the T_R and $CD25^-$ TCR repertoires. First, we asked whether TCRs present at high frequency in the $CD25^+$ sample set were equally represented in the corresponding $CD25^-$ set, and vice versa (Table 3). We found that the vast majority of these TCRs were asymmetrically distributed between the $CD25^+$ and $CD25^-$ subsets. This finding is further supported by the lack of overlap between sequences found in both T_R TCRα sets ($n = 11$) and sequences found in both $CD25^-$ TCRα sets ($n = 19$). We reasoned that sequences found in both independently derived T_R or both $CD25^-$ TCRα libraries should best represent the TCR repertoire of the respective T cell subset. Finally, we utilized two different statistical estimators of similarity between two populations. One of these estimators, the Jaccard similarity index, provides an estimate of overlap between two TCR repertoires independently of the number of repeat sequences observed within each subset (Figure 2C). In contrast, the Morisita-Horn index incorporates both unique and repeat sequence information (Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/21/2/267/DC1). Nevertheless, both approaches provide comparable semiquantitative estimates of a less than 25% overlap between the T_R and $CD25^-$ TCR repertoires. A logical implication of this finding is that certain TCR sequences may skew T cell development toward the T_R or $CD25^-$ phenotype, and the question arises as to the role of TCR specificity in T_R immunobiology.

Table 3. Distribution of TCRα Chains Found at High Frequencies

Unique TCRα Clone	$CD25^+$	$CD25^-$
Experiment 1		
G69	5	0
G25	5	0
R19	4	0
R18	4	0
B31	2	5
L35	0	4
L69	0	4
L33	0	5
B33	0	5
B2	0	6
Experiment 2		
G8	5	0
G43	7	4
B2	0	4
B54	1	5
B15	1	5
B33	0	7
B8	1	8
B5	1	14

TCRα clones in italics were tested individually in experiments described in Figure 5. TCRα clones found at >3% frequency are shown.

T_R, but Not $CD25^-$, $CD4^+$ T Cells, Contain a High Frequency of Self-Reactive TCRs

Next, we investigated the functional implications of the differences observed between the T_R and $CD25^-$ TCR repertoire by sequencing. A hallmark of naturally arising $CD25^+$ $CD4^+$ T regulatory cells is their inability to proliferate or produce IL-2 upon TCR stimulation in vitro (Gavin et al., 2001; Takahashi et al., 1998; Thornton and Shevach, 1998). This property of T_R hinders the study of their TCR specificity. To overcome this problem, we retrovirally transferred T_R TCRα chains into conventional $CD25^-$ $CD4^+$ recipient T cells (Figure 1). We reasoned that activation through the transferred T_R TCR should elicit conventional responses in the transduced T cells, such as proliferation.

We chose TCli-$\alpha\beta$ $\times$ $RAG1^{-/-}$ (Vα18, Vβ6) mice described above as the source of conventional $CD25^-$ $CD4^+$ T cells. These T cells have a single defined specificity to a foreign antigen, human CLIP, and do not express endogenous TCRα chains. Upon retroviral transduction of these cells, the level of retroviral LTR-driven TRAV14 TCRα expression was comparable to that of endogenous TCRα expression in TCli-$\alpha\beta$ $\times$ $RAG1^{+/+}$ T cells (Supplemental Figure S2) and stable upon adoptive transfer into lymphopenic hosts for up to 3 months (data not shown).

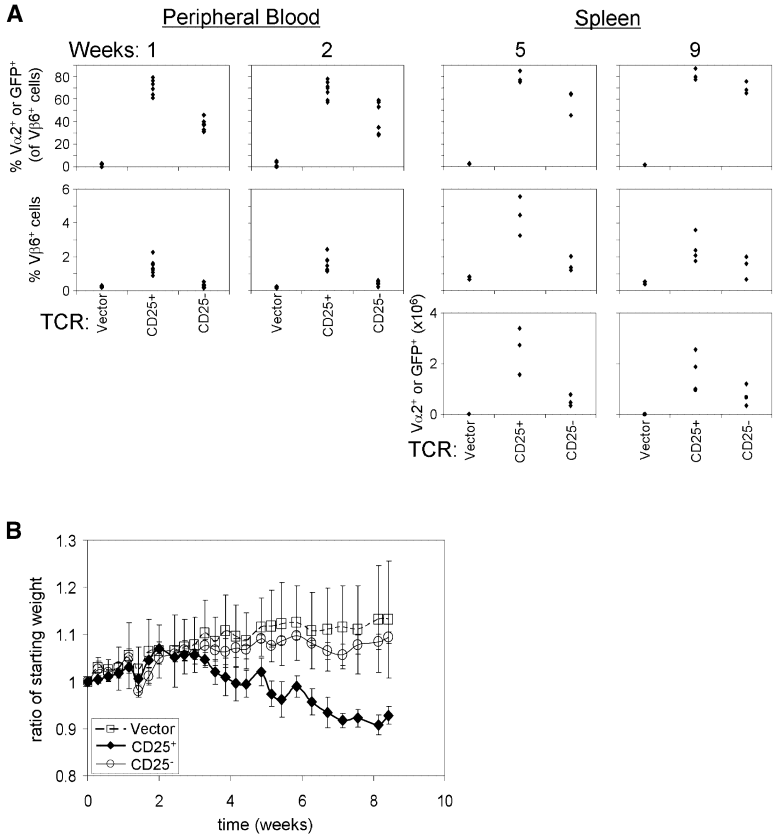

Figure 3. T_R TCRs Are Self-Reactive: Acquisition of T_R TCRs Results in Rapid In Vivo Expansion and Wasting Disease

TCli αβ-TCR × RAG1-deficient $CD4^+$ T cells were retrovirally transduced with MigR1 "empty" vector or with TRAV14 TCRα libraries derived from $CD25^+$ or $CD25^-$ $CD4^+$ T cells. Three days after in vitro activation, 10^6 T cells were adoptively transferred into TCRα-deficient hosts by intravenous injection. Transduction efficiency was approximately 1% for all conditions in this experiment.

(A) Expansion of TCRα-transduced T cells. The relative expansion of transduced T cells was assessed as the percentage of $V\alpha2^+$ or GFP^+ cells within the adoptively transferred $V\beta6^+$ T cell population in the peripheral blood and the spleen at the times indicated (top). The size of the adoptively transferred population is also shown as the percentage of $V\beta6^+$ T cells in the peripheral blood or spleen (middle). Last, the absolute numbers of transduced T cells residing in the spleen are calculated based on flow cytometric analysis (bottom). Each point represents data from an individual mouse ("empty" vector n = 4, $CD25^+$ n = 7, $CD25^-$ n = 6), with half of the group harvested at 5 or 9 weeks.

(B) Expansion of T cells expressing $CD25^+$ TCRα chains causes wasting disease. The ratio of the starting weight of recipient mice is shown ± SEM ("empty" vector n = 2, $CD25^+$ n = 4, $CD25^-$ n = 3). Weight loss was associated with gross pathologic findings of colon inflammation, including bowel wall thickening, poorly formed stool, and shortening of the colon (data not shown).

Using this retroviral transduction system, we directly tested the hypothesis that T_R TCRs have a higher avidity for self-antigens than $CD25^-$ TCRs by assessing the ability of these TCRs to confer the capacity to expand in vivo in syngeneic lymphopenic hosts. For these experiments, we intentionally chose retroviral infection conditions that resulted in a low transduction efficiency to maximize the dynamic range of relative expansion of transduced versus nontransduced T cells. Since we did not know a priori the avidity of naturally arising $CD25^+$ TCRs for self-peptide:MHC class II complexes, we selected T cell expansion in lymphopenic hosts as the most sensitive assay for in vivo assessment of self-reactivity. It has been shown for naive and memory T cells that the rate and extent of homeostatic expansion in lymphopenic hosts is dependent upon the relative avidity of TCR for self-peptide:MHC class II complexes, including that of nonpathogenic TCR-ligand interactions (Kassiotis et al., 2003; Moses et al., 2003).

For our initial experiments, we transduced complex structures of TCRs from T_R or $CD25^-$ $CD4^+$ T cell-derived TRAV14 TCRα libraries. On day 3 after in vitro activation, we adoptively transferred the population containing both transduced and nontransduced TCli-αβ $RAG1^{-/-}$ T cells into $TCR\alpha^{-/-}$ hosts to assess the relative expansion in vivo of T cells expressing the retroviral TCR. We found a striking expansion of T cells transduced with T_R TCRs relative to the cotransferred nontransduced T cells as early as day 8 after transfer, while T cells transduced with "empty" vector showed little relative expansion (Figure 3A). T cells transduced with $CD25^-$ TCRα's also expanded, but with significantly delayed kinetics in comparison to T cells expressing T_R TCRs. Expression of some self-reactive TCRs within the $CD25^-$ subset is not surprising as it has been well documented previously (Powrie et al., 1993; Sakaguchi et al., 1995). Increases in total T cell numbers correlated with the relative expansion of the transduced T cells. Of note, we did not find substantial expansion of "empty" vector-transduced T cells, suggesting that the TCli TCR does not facilitate strong homeostatic expansion. In addition to conferring the ability to expand in vivo, T_R TCR expression resulted in wasting disease reflected by weight loss during the course of the experiment (Figure 3B). In contrast, expression of $CD25^-$ TCRs or "empty" vector did not result in weight loss. Taken together, these data suggest that at the population level, T_R TCRs have substantially more efficient interactions with self-peptide:MHC class II complexes in comparison with $CD25^-$ $CD4^+$ TCRs. Because the self-peptide ligands for $CD25^+$ TCRs have not been identified, these TCRs may either have increased affinity for self-peptide:MHC class II complexes and/or recognize more abundant self-peptides, which would collectively result in an increased avidity for self ligands.

Consistent with the ability of T cells expressing T_R TCRs to cause pathologic weight loss, in vitro analysis of these T cells isolated from the recipient mice showed

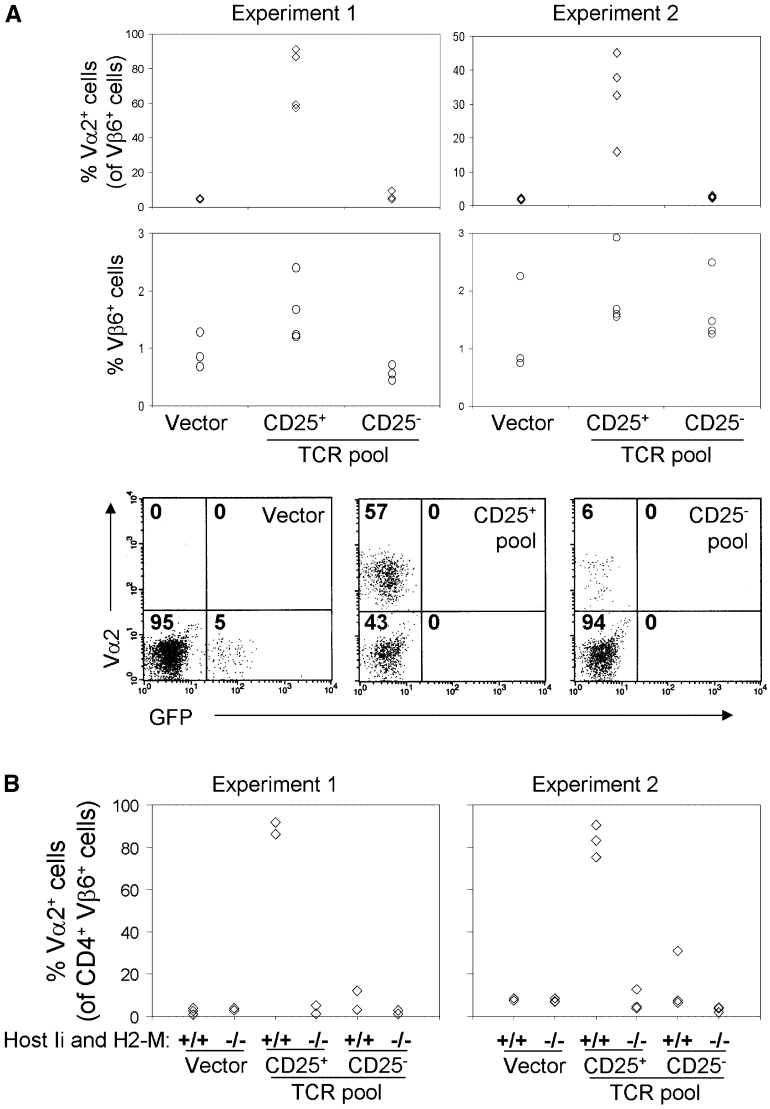

Figure 4. A Pool of 10 T_R TCRs Recognize Peripherally Presented Self Antigen:MHC Class II Complexes with Higher Avidity than 10 CD25$^-$ TCRs

(A) Analysis of a pool of 10 CD25$^+$ or CD25$^-$ TCRα chains (Supplemental Table S1) shared between both independently derived CD25$^+$ TCRα libraries or both CD25$^-$ TCRα libraries, respectively. TCli $\alpha\beta$-TCR $\times$ RAG1-deficient T cells were retrovirally transduced with T_R or CD25$^-$ TCRα genes in MigR1-MG (without IRES-GFP), or "empty" vector MigR1. Two days later, 4×10^5 T cells were adoptively transferred into RAG1-deficient hosts for 11 days. The percentage of transduced T cells in the population adoptively transferred was normalized to a fixed value (between 3% and 6%) using concurrently activated nontransduced TCli $\alpha\beta$-TCR $\times$ RAG1-deficient transgenic T cells. Expansion of T cells expressing transduced TCRs (the percentage of Vα2$^+$ or GFP$^+$ cells within the Vβ6$^+$ CD4$^+$ T cell population, top) and the total percentage of transferred T cells in the spleen (% Vβ6$^+$ CD4$^+$, middle) was assessed by flow cytometry. Bottom panels show representative FACS plots of the graphed data. Two independent experiments are shown. Each symbol represents relative cell expansion in a single recipient.

(B) CD25$^+$ TCR recognition of self peptide:MHC class II complexes is required for in vivo expansion. TCli $\alpha\beta$-TCR $\times$ RAG1-deficient T cells were retrovirally transduced as above. Two days later, 4×10^5 T cells were adoptively transferred into TCRα-deficient or TCRα $\times$ Ii $\times$ H-2M triple-deficient hosts for 12 days before assessment of expansion of transduced T cells in the spleen by flow cytometry as the percentage of Vα2$^+$ or GFP$^+$ cells within the total transferred CD4$^+$ Vβ6$^+$ T cell population. The percentage of transduced T cells in the adoptively transferred population was normalized to 2.33% or 5% using concurrently activated nontransduced TCli $\alpha\beta$-TCR $\times$ RAG1-deficient T cells.

that they were neither anergic nor suppressive (data not shown). There was also no expression of CD25 by T cells transduced with either T_R or CD25$^-$ TCRα's at the time of analysis (data not shown). Thus, at a population level, acquisition of T_R TCR specificity by activated conventional CD25$^-$ T cells is not capable of conferring a T_R phenotype.

The rapid in vivo expansion of T cells transduced with T_R TCRs suggested that the frequency of self-reactive TCRs was high. To directly test this hypothesis, we decided to analyze a pool of only 10 unique TCRα chains from the T_R or CD25$^-$ subset. For these experiments, we selected TCRα chains shared between the two independently derived T_R TCRα sequence sets or the two CD25$^-$ TCRα sets. We reasoned that utilization of these TCRs is reliably skewed toward the T_R or CD25$^-$ T cell subsets and that the CD25$^+$ TCRs are therefore less likely to be contaminated with TCRs derived from activated CD25$^-$ CD4$^+$ T cells. These TCRs were diverse and not dominated by a particular sequence motif at the TRAV, TRAJ, or CDR3 level (Supplemental Table S1). Using the same retroviral transduction protocol, we found that the T_R TCR pool conferred the capacity to expand in RAG1-deficient mice when transduced into TCli-$\alpha\beta$ $\times$ RAG1$^{-/-}$ T cells (Figure 4A). Similar T cell expansion conferred by CD25$^+$ TCRs was also observed in TCRα-deficient mice, but not in TCRα $\times$ Ii $\times$ H-2M triple deficient recipients (Figure 4B), which display a greatly diminished repertoire of self-peptide:MHC class II complexes (Kovats et al., 1998). Thus, the observed expansion of CD25$^+$ TCR expressing TCli-$\alpha\beta$ $\times$ RAG1$^{-/-}$ T cells is dependent upon recognition of self-peptide:MHC class II complexes. Notably, we did not observe substantial in vivo expansion of T cells expressing the pool of CD25$^-$ TCRs, suggesting that the frequency of self-reactive CD25$^-$ TCRs responsible for in vivo expansion observed with a complex set of TCRs, as shown above in Figure 4A, is quite low. Thus, these data confirmed that CD25$^+$ TCRs have increased avidity for self ligands compared with CD25$^-$ TCRs.

We then analyzed the individual TCRs in these pools. We did find that certain TCRs were poorly recognized by the Vα2 monoclonal antibody, presumably due to their TRAV14 subtype. Thus, additional TCRs were in-

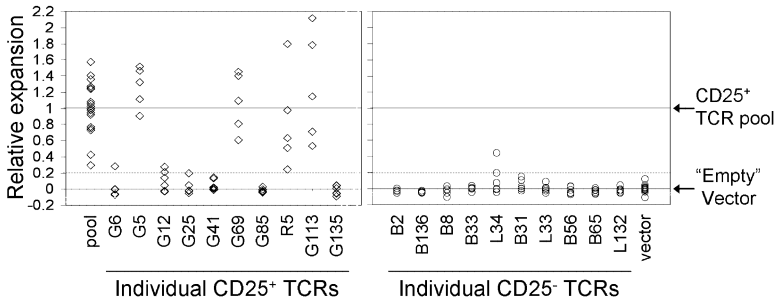

Figure 5. A High Frequency of Individual T_R, but Not CD25$^-$, TCRs Recognize Self Peptide:MHC Class II Complexes with Increased Avidity

TCRs used in Figure 4 were individually tested using the same approach. Because some of these TCRs were poorly recognized by the Vα2 antibody, an additional CD25$^+$ and 3 additional CD25$^-$ TCRs were also analyzed to make a group of 10 individual TCRs tested for each subset (Supplemental Table S1). The plots summarize data from eight different experiments, with each symbol representing data from a single recipient. In every experiment, T cells transduced with the pool of CD25$^+$ TCRs were used as the positive control and with "empty" vector as the negative control. To normalize the expansion of transduced T cells between experiments, we used the following formula: relative expansion = (% experimental − % "empty" vector)/(% CD25$^+$ pool − % "empty" vector). Thus, expansion equivalent to the pool of 10 CD25$^+$ TCRs would give a value of 1, and no expansion a value of 0. The dashed line at 0.2 represents the upper limit of variability found in the "empty" vector controls. Application of the nonparametric Wilcoxon Rank Sum Test to the mean relative expansion for each TCR demonstrates that the differences observed between the groups of CD25$^+$ and CD25$^-$ TCRs tested are statistically significant ($p \leq 0.009$).

cluded in the analysis such that a total 10 CD25$^+$ and 10 CD25$^-$ TCRs were tested individually (Supplemental Table S1). Remarkably, we found that approximately 60% (6/10) of T_R TCRs tested were capable of conferring some ability to expand in vivo (Figure 5). Four of these TCRs reproducibly led to a rapid expansion, whereas the remaining two conferred only a modest expansion of transduced T cells in some of the recipient mice. In contrast to the T_R TCRs, only 1 of 10 CD25$^-$ TCRs showed any sign of self-reactivity in 2 of 5 recipients. None of the CD25$^-$ TCRs showed the ability to confer rapid in vivo expansion in all recipient mice. Thus, these data directly demonstrate that naturally arising CD25$^+$, but not CD25$^-$, T cell populations display a high frequency of TCRs with higher avidity for peripheral self-antigens.

CD25$^+$ TCRs Can Recognize Self Peptides Presented by Splenic APCs

Expansion of T cells bearing T_R TCRs in vivo may result from TCR interaction with tissue-specific or ubiquitously presented antigens. Previous indirect studies have suggested the existence of naturally arising tissue-specific T_R (Seddon and Mason, 1998; Taguchi et al., 1994) but do not exclude the possibility of T_R responsive to ubiquitously expressed antigen. Thus, we tested whether T_R TCRs could recognize self peptides presented on splenic APCs. We found that only T cells retrovirally transduced with T_R, but not CD25$^-$ TCRs, proliferated in response to autologous splenic APCs (Supplemental Figure S3A). Furthermore, these T cells also responded, albeit at a lower level, to invariant chain-deficient APCs (Ii^0), which primarily present peptides derived from endogenously synthesized proteins that are ubiquitously expressed (Kovats et al., 1998). We also found reactivity to self peptides in the limited pool of 10 TCRs found in both independently derived TCR sets described above (Supplemental Figure S3B). When tested individually, we found that it was 1 of the 4 in vivo reactive CD25$^+$ TCRs (Figure 5) that conferred the ability to proliferate in vitro to autologous splenic APCs (data not shown). This is likely due to the relative insensitivity of the in vitro proliferation assay, which assesses cells entering S phase over an 18 hr period, in comparison with in vivo expansion, which measures accumulation of T cells over 11 day period or more in a lymphopenic environment favoring cell growth. Thus, these data suggest that a sizable subset of T_R can recognize ubiquitously presented self antigens, which is further supported by our finding that 10%–20% of normal polyclonal CD25$^+$ T_R maintain CD25 expression in response to autologous APCs in vitro (Supplemental Figure S4).

Self-Reactive CD25$^+$ TCRs Skew CD4$^+$ T Cell Development or Survival toward the CD25$^+$ Phenotype

Both the in vitro and in vivo data directly demonstrate that a large percentage of TCRs derived from CD25$^+$ T cells interact with self ligands with greater avidity than the TCRs derived from CD25$^-$ T cells (Figures 3–5). However, it can not be ascertained from this data what the functional implications of these differences in self-reactivity are. We hypothesized that the observed increase in self-reactivity of CD25$^+$-derived TCRs would skew T cell development toward the CD25$^+$ phenotype, which would be consistent with studies using TCR transgenic mice (Jordan et al., 2001; Klein et al., 2003; Walker et al., 2003a) and with our sequencing data suggesting that the CD25$^+$ and CD25$^-$ TCR repertoires are only partially overlapping (Figure 2C; Supplemental Figure S1). Thus, we retrovirally transduced TRAV14 TCRα chains into TCli TCRβ transgenic hematopoetic bone marrow (BM) progenitors, which were adoptively transferred into lethally irradiated hosts (Figure 6). We analyzed three self-reactive CD25$^+$ TCRs (S19, R5, and G113) and three nonreactive CD25$^-$ TCRs (N11, B2, and B8). The percentage of peripheral CD4$^+$ T cells expressing the retroviral TCRα appeared comparable between the CD25$^+$ and CD25$^-$ TCRs except for S19, which was decreased likely due to partial deletion. We found that all three self-reactive CD25$^+$-derived TCRs favored the development or survival of CD25-expressing cells in the peripheral lymph nodes, albeit with some variability ($\sim$6%–50% CD25$^+$). In contrast, none of the three non-self-reactive CD25$^-$-derived TCRs were capable of generating any substantial numbers of CD25$^+$ T cells ($<$0.5%). Although further studies are required, these findings suggest that the increased self-reactivity observed in CD25$^+$ TCRs has functional implications for the generation of CD25-expressing T cells.

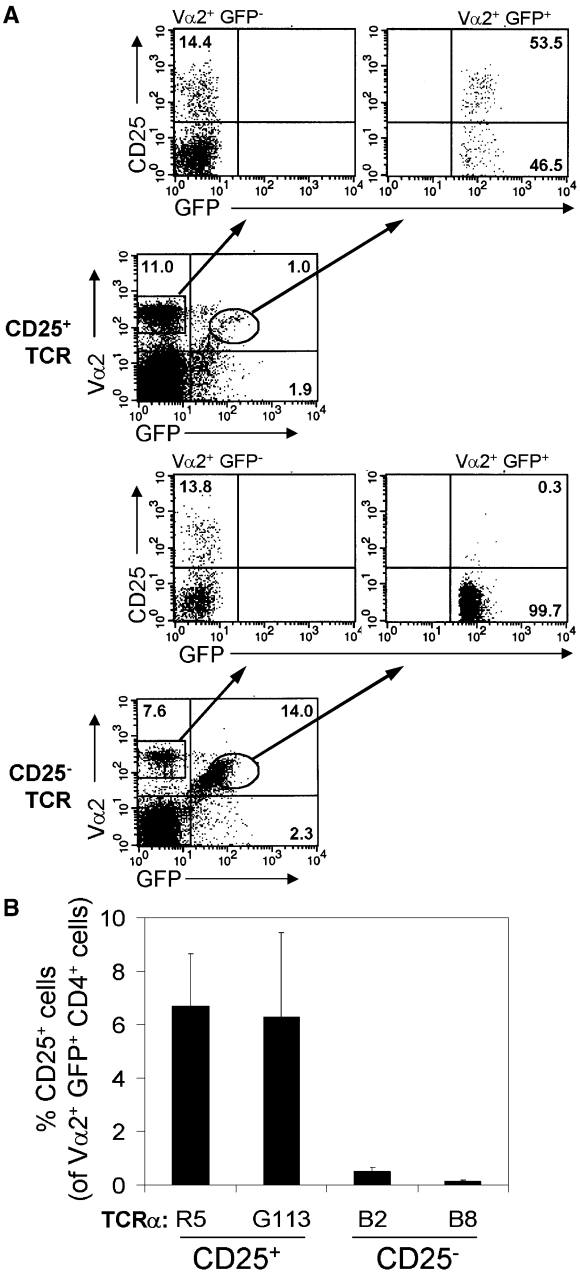

Figure 6. Skewing of CD25$^+$ T Cell Development by TCR Specificity

(A) Analysis of CD25$^+$ and CD25$^-$ CD4$^+$ T cell subsets in bone marrow chimeras expressing retrovirally transduced TCRα clones S19 and N11. The CD25$^+$-derived clone S19, but not the CD25$^-$-derived clone N11, was found to confer in vivo self-reactivity in experiments similar to those shown in Figure 4 (data not shown). These TCRα genes were retrovirally transduced into TCli TCR-β bone marrow and adoptively transferred to lethally irradiated RAG1-deficient mice as previously described (Gough and Raines, 2003). After 6 weeks, expression of CD25, GFP, and Vα2 was assessed by flow cytometry of axillary and inguinal lymph node cells. Cells analyzed were gated for CD4 expression. T cells expressing the retroviral TRAV14 TCRα gene co-express GFP present in the MigR1 vector. Similar results were obtained by flow cytometric analysis of splenocytes from chimeric mice (data not shown).

(B) Analysis of CD25$^+$ and CD25$^-$ CD4$^+$ T cell subsets in bone marrow chimeras expressing retrovirally transduced TCRα clones R5, G113, B2, and B8. The self-reactive CD25$^+$-derived TCRα clones R5 and G113 and the non-self-reactive CD25$^-$-derived TCRα clones B2 and B8 (Figure 5) were individually transduced into TCli-β transgenic bone marrow as above. The percentage of CD25 expressing retrovirally transduced Vα2$^+$ GFP$^+$ CD4$^+$ T cells from the axillary and inguinal lymph nodes 6 weeks after bone marrow reconstitution is shown ($\pm$SEM). Data are pooled from two independent experiments (R5 n = 6, G113 n = 3, B2 n = 5, B8 n = 2).

Discussion

Characterization of the T_R TCR repertoire is important for understanding the development and function of naturally arising regulatory T cells. Here, we address this issue by combining two previously described approaches: (1) the use of a fixed TCRβ chain to limit the TCR repertoire to allow for the analysis of TCRs at the level of individual sequences (Correia-Neves et al., 2001; Sant'Angelo et al., 1997); and (2) the use of retroviral transduction to introduce TCR genes into CD4$^+$ T cells (Fujio et al., 2000). This strategy allowed us to correlate the T_R and CD25$^-$ CD4$^+$ TCR sequence information with a functional assessment of their TCR specificity.

Analysis of the T_R TCR repertoire based on the sampling of TRAV14:TCli-TCRβ TCRs by direct sequencing of TCRα chains suggested that the T_R TCR repertoire is as diverse as the CD25$^-$ repertoire at the level of CDR3 amino acid sequences. This is an unexpected finding, as the CD25$^+$ CD4$^+$ T cell population is $\sim$5%–10% of the CD25$^-$ population. Although further studies are needed to extend this observation, it raises several interesting issues regarding T_R biology. One possible explanation for equivalent TCR diversities in peripheral T cell populations is that the postthymic selection CD25$^-$ CD4$^+$ TCR repertoire is subjected to further restriction in the periphery due to a requirement for low-avidity TCR interactions with self peptides bound to MHC class II molecules. Experimental support for this notion comes from a recent analysis of mice with a restricted TCR repertoire, TCRα minilocus mice (Correia-Neves et al., 2001). In contrast, higher avidity interactions of T_R TCRs with self peptide:MHC class II complexes would lead to a lesser restriction of the postthymic TCR repertoire. As illustrated by our adoptive transfer experiments, these peripheral T_R ligands are readily available (Figures 3–5).

The equivalent diversity of T_R and CD25$^-$ TCR repertoires also suggests that T_R can recognize a wide variety of antigens. This may reconcile our finding of increased avidity of T_R for self ligands with recent reports showing expansion of naturally occurring T_R upon recognition of, and suppression of immune responses to, *Leishmania major* and other pathogens (Belkaid et al., 2002; Montagnoli et al., 2002).

Our sequencing data also suggest that the T_R and CD25$^-$ TCR repertoires have only a minor overlap (Figure 2C; Supplemental Figure S1). Although the differences between the TCR repertoires are consistent with the observed enhanced self-reactivity with self ligands of CD25$^+$, but not CD25$^-$, TCRs (Figure 5), the cause of the overlap between these two TCR repertoires is less clear. This overlap is unlikely to result from a contamination during cell isolation, as the purity was >95% and >98% for the CD25$^+$ and CD25$^-$ subsets, respectively. Alternatively, it may be explained by the presence

of recently activated CD25$^-$ T cells in the CD25$^+$ subset, or the presence of regulatory T cells in both CD25$^+$ and CD25$^-$ subsets, such as GITR$^+$ CD25$^-$ regulatory T cells (Uraushihara et al., 2003). However, this is an unlikely possibility as our preliminary analysis of GFP-Foxp3 fusion protein knockin mice revealed that more than 95% of CD25$^+$ CD4$^+$ T cells express Foxp3 within the gate we used for sorting (J.D. Fontenot and A.Y.R., unpublished observations; data not shown). This suggests that our FACS-sorted CD25$^+$ T cell populations contain very few nonregulatory T cells.

Another likely cause for the overlap between the T_R and CD25$^-$ TCR repertoires may relate to the mechanisms that control T_R development in the thymus. For example, developing T cells may encounter a T_R selecting ligand with only a certain probability or may stochastically express a gene(s) critical for T_R development, such as Foxp3 (Fontenot et al., 2003). Alternatively, it may reflect the need for limiting non-TCR-derived signal(s). These non-mutually exclusive hypotheses are consistent with the report that only ~50% of TCR transgenic T cells develop into T_R, despite ubiquitous cognate antigen expression in the thymus (Jordan et al., 2001).

We have employed in vivo expansion in lymphopenic hosts as the most sensitive assay for TCR interactions with self ligands. The avidity range of these interactions may vary from relatively weak ("homeostatic") to high ("autoreactive"), although the threshold between these levels cannot be easily distinguished. We use the term avidity here to represent the aggregate efficiency of TCR-ligand interactions. Because the self ligands recognized by the CD25$^+$ TCRs are unknown, we cannot discriminate between the contribution of increased TCR affinity or higher self-peptide ligand abundance to the overall TCR signal. The lack of expansion conferred by individual CD25$^-$ TCRs (Figure 5) suggests a low frequency of high-avidity TCR in this subset of cells compared with the T_R subset. We do believe that some of the T_R TCRs are autoreactive, as T cells transduced with a complex mixture of CD25$^+$ TCRs cause wasting disease in lymphopenic hosts (Figure 3B). We have not, however, observed in vivo expansion of transduced T cells in nonlymphopenic Ly5.1$^+$ hosts (data not shown), suggesting that their expansion is suppressed by endogenous T_R and/or their autoimmune potential is facilitated by a lymphopenic environment (McHugh and Shevach, 2002). Thus, our findings suggest that the avidity of the majority of T_R TCRs for self ligands is below the range characteristic of conventional T cell responses to cognate antigens. This is consistent with the fact that peripheral T_R must have escaped negative selection in the thymus.

Nonetheless, the differences that we observe in the self-reactivity between CD25$^+$ and CD25$^-$ TCRs using in vivo expansion in a lymphopenic hosts do appear to have functional consequences for T cell development (Figure 6). While a full evaluation of the role of TCR specificity on regulatory T cell development is beyond the scope of this manuscript, we do observe that individual self-reactive CD25$^+$, but not nonreactive CD25$^-$, TCRs facilitate generation, albeit at a varying degree, of CD25-expressing T cells. However, the existing data do not allow us to determine whether these naturally arising TCRs are actively dictating a program of regulatory T cell development or allowing precommitted regulatory T cells to expand or survive.

While our experiments confirmed the ability of T_R TCRs to recognize self antigens, we observed a substantial variation in the efficiency of interaction with self ligands among individual TCRs (Figure 5). Although a large proportion (4/10) of these TCRs was strongly self-reactive in vivo, the others were only intermittently and moderately self-reactive or nonreactive as measured by T cell expansion in lymphopenic hosts. One possible interpretation of this result is that the latter TCR group falls into the lower range of T_R TCR avidity for self ligands that can not be detected by our assay. Another possibility is that self-antigen presentation to some T_R TCRs can be spatially restricted such that the transferred T cells do not gain sufficient access to these sites during the 11 day window in our protocol. For example, adoptively transferred T cells expressing low levels of CD62L may have limited trafficking through lymph nodes associated with a particular organ, which serve as sites of presentation of tissue-specific antigens by dendritic cells (Scheinecker et al., 2002).

While the specific peptide ligands recognized by T_R TCRs remain to be identified, initial characterization of their specificity has suggested that T_R TCRs recognize MHC class II in a self-peptide-dependent manner, as T_R TCRs failed to recognize in vitro APCs from previously published CD22-dbl^o mice expressing primarily a single CD22 peptide:MHC class II complex (data not shown; Barton et al., 2002). T_R TCRs also failed to facilitate in vivo expansion in TCR$\alpha \times$ Ii $\times$ H-2M mice (Figure 4B), which display a greatly reduced self peptide:MHC class II repertoire (Kovats et al., 1998). What proteins are these T_R-reactive peptides derived from? Our analysis of proliferative in vitro responses of TCIi-$\alpha\beta$ RAG-deficient T cells transduced with T_R TRAV14 TCRα chain libraries showed reactivity to wild-type syngeneic MHC class II-positive APCs, but also to Ii-deficient APCs, albeit at a reduced level (Supplemental Figure S3A). Although we cannot formally exclude the possibility that T_R TCRs may recognize antigens derived from commensal or pathogenic flora or viruses, we believe that splenic APCs from B6 mice housed under strict SPF conditions are unlikely to harbor class II bound peptides of microbial or viral origin. Furthermore, Ii^0 APCs are deficient in the presentation of exogenous protein antigens, suggesting that some T_R TCRs may recognize peptides derived from endogenously synthesized proteins displayed on Ii^0 APCs (Kovats et al., 1998). The recognition of ubiquitously expressed self peptides by some T_R TCRs does not exclude recognition of tissue-specific peptides by others. In fact, we have found that adoptive transfer of TCIi-$\alpha\beta$ RAG$^{-/-}$ T cells transduced with a combination of four CD25$^+$ TCRs leads to peribronchiolitis and vasculitis in the lungs of lymphopenic RAG1-deficient hosts (data not shown). Although we cannot exclude the possibility of ubiquitous antigen reactivity resulting in a tissue-specific pathology (Matsumoto et al., 1999), these data are suggestive of tissue-specific T_R TCRs.

In summary, we have found that the CD25$^+$ CD4$^+$ T_R TCR repertoire is as diverse as CD25$^-$ CD4$^+$ TCR repertoire. By using TCR gene transfer, we have functionally assessed T_R self-reactivity at a single TCR level and found that the CD25$^+$, but not CD25$^-$, CD4$^+$ T cell

subset exhibits a high frequency of TCRs with enhanced avidity against self peptides bound to MHC class II molecules expressed by peripheral APCs. This is consistent with the largely nonoverlapping TCR repertoires observed in these T cell subsets. This result further suggests that a large proportion of naturally arising T_R recognize constitutively presented self ligands and is thus chronically stimulated. We speculate that this may be important for providing a basal threshold for immune activation, which may then be dynamically regulated by the level of presentation of T_R-specific self-antigens.

Experimental Procedures

Mice

Mice were housed under specific pathogen-free conditions and used according to the guidelines of the Institutional Animal Care Committee at the University of Washington. MHC class II-, TCRα-, invariant chain- (Ii^0), TCR$\beta\delta$-, and RAG1-deficient mice were obtained from the Jackson Laboratory (Bar Harbor, ME). C57Bl/6 mice were obtained from Charles River (Wilmington, MA). TCIi $\alpha\beta$-TCR (Vα18, Vβ6) and TCRβ (Vβ6) only transgenic mice were previously described (Wong et al., 2000).

Reagents

DMEM containing 10% fetal calf serum, 2-ME, penicillin/streptomycin, sodium pyruvate, L-glutamine, and HEPES (all Life Technologies, Gaithersburg, PA) was used as tissue culture medium. Biotin- (BIO), fluorescein isothiocyanate- (FITC), phycoerythrin- (PE), peridinin-chlorophyll protein (PerCP), or allophycocyanin- (APC) conjugated antibodies to Vα2 (B20.1) and Vβ6 (RR4-7) were obtained from Pharmingen (San Diego, CA); conjugated antibodies to CD4 (RM4-5) and CD25 (PC61) were obtained from eBioscience (San Diego, CA). Flow cytometry utilized a FACSCalibur (BD Biosciences, San Jose, CA).

T Cell Isolation

Initial cell purification was performed from the spleen and lymph nodes of two mice using anti-CD25-biotin, anti-biotin microbeads, and an AutoMACS magnetic cell sorter (Miltenyi, Bergisch Gladbach, Germany). Positively sorted cells were stained with APC-conjugated anti-CD25, PE-conjugated anti-Vβ6, and anti-CD4-FITC antibodies. $CD25^+$ $CD4^+$ $V\beta6^+$ T cells were then sorted using a FACSVantage flow cytometer (BD Biosciences). Negatively sorted cells were further purified with anti-CD4-microbeads and AutoMACS (Miltenyi), followed by FACS sorting for $CD25^-$ $CD4^+$ $V\beta6^+$ T cells. For experiment #1, 2×10^5 $CD25^+$ and 1.8×10^6 $CD25^-$ T cells were sorted. For experiment #2, 5×10^5 $CD25^+$ and 2×10^6 $CD25^-$ T cells were used.

TCRα Libraries

RNA from purified T cell populations was generated using RNeasy (Qiagen, Valencia, CA). cDNA was synthesized using Thermoscript reverse transcriptase (Life Technologies) with oligo-dT priming. TRAV14 TCRα chains were then amplified by high-fidelity PCR (Advantage 2 HF, BD Biosciences) using the following primers: sense, ATATCTCGAGATGGACAAGATCCTGACAGCA; and antisense, CAC GAATTCTCAGTCTTGCAGACCTCAACT. The PCR products were digested with XhoI-EcoR1 for cloning into MigR1 (Pear et al., 1998), which utilizes an IRES-GFP reporter (internal ribosome entry site), or into MigR1-MG (minus IRES-GFP). MigR1-MG was derived from MigR1 by replacing the IRES-GFP with the corresponding EcoRI-SalI fragment of pBLUESCRIPT (Stratagene, La Jolla, CA). TCRα clones were sequenced using the BigDye kit (Applied Biosystems, Foster City, CA) and analyzed by IMGT-V-QUEST. TCR sequences were discarded if the V-region score was <1200. The error rate from RT-PCR was 10 errors in 106 Cα regions sequenced, equivalent to $\sim$1:4000 bases or $\sim$1 per 100 CDR3 based on an average 13 aa length.

Retroviral Transduction

MigR1-based retroviral vectors were transfected into the ϕNX-E packaging cell line (Pear et al., 1993) using FuGENE 6 (Roche, Indianapolis, IN). Culture medium was changed the following day, and virus was collected after culturing cells at 32°C for 24–30 hr. TCli$\alpha\beta \times$ RAG1° spleen and lymph node cells were stimulated for 24–30 hr with 0.5–1 μg ml^{-1} of human CLIP in the presence of 20 U ml^{-1} human IL-2 (Roche) prior to a "spinfection" at 2500 RPM, 37°C, for 90 min with 0.45 μm filtered retroviral supernatant supplemented with 4 μg ml^{-1} polybrene (Sigma, St. Louis, MO), IL-2 (20 U ml^{-1}), and HEPES 0.01 M. For T cells slated for in vivo experiments for relative expansion in Figures 4 and 5, less retroviral supernatant was used to diminish the transduction efficiency. Cells were then cultured in the presence of retroviral supernatant for another day, washed, and resuspended in culture medium containing 20 U ml^{-1} IL-2.

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